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Reduced cAMP Secretion in *Dictyostelium discoideum* Mutant HB3

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Extracellular cAMP induces the intracellular synthesis and subsequent secretion of cAMP in *Dictyostelium discoideum* (relay). cAMP relay was strongly diminished in mutant HB3 which shows abnormal development by making very small fruiting bodies. Extracellular cAMP binds to receptors on the surface of mutant cells and induces the rapid activation of adenylate cyclase. Intracellular cAMP rises to a concentration as high as that in wild-type cells but only a very small amount of cAMP is secreted. cAMP secretion in wild-type cells starts immediately after cAMP production, and is proportional to the intracellular cAMP concentration. In the mutant cells cAMP secretion starts a few minutes after cAMP production; by that time most of the intracellular cAMP is already degraded by phosphodiesterase and little cAMP is available for secretion. We conclude that mutant HB3 has a defect in the mechanism by which *Dictyostelium* cells secrete cAMP.

INTRODUCTION

Extracellular cAMP binds to surface receptors of *Dictyostelium discoideum* cells and induces several intracellular responses which ultimately lead to chemotaxis, cell aggregation, and the formation of a fruiting body (Devreotes, 1983; Gerisch, 1987; Janssens and Van Haastert, 1987). The activation of adenylate cyclase is one of these responses (Roos and Gerisch, 1976).

About half of the produced cAMP is degraded intracellularly and the other half is secreted (Dinauer *et al.*, 1980a), where it may activate receptors on other cells. This relay mechanism, combined with cAMP-induced chemotaxis, ensures the attraction of amoebae from a large area to central collecting points. Binding of cAMP to surface receptors induces the activation of adenylate cyclase with a short lag period of about 30 sec. Enzyme activity is maximal after about 1-2 min (Dinauer *et al.*, 1980b), and then declines to basal levels by an adaptation mechanism (Dinauer *et al.*, 1980c). It has been proposed that activation of adenylate cyclase proceeds via a guanine nucleotide binding protein (G protein) and that adaptation is mediated by the uncoupling of the receptor-G protein interaction (Theibert and Devreotes, 1986; Van Haastert *et al.*, 1987) due to phosphorylation of the receptor (Klein *et al.*, 1986; Klein *et al.*, 1987). The intracellular cAMP concentration is maximal after about 1-2 min, and then declines by multiple reactions, i.e., by adaptation of adenylate cyclase, by intracellular degradation of cAMP, and by secretion of cAMP.

Besides the function during cell aggregation, extracellular cAMP also plays an important role during the multicellular stage in the differentiation of prestalk and prespore cells (Chisholm *et al.*, 1984; Kay, 1982; Mehdy and Firtel, 1985), and in the coordination of cell movement in the multicellular stage (Schaap *et al.*, 1984). These conclusions are partly derived from mutant studies, notably mutant N7 (new name *synag 7*). This mutant does not show cAMP relay because cAMP cannot activate adenylate cyclase, and consequently cells do not aggregate (Schaap *et al.*, 1986; Theibert and Devreotes, 1986; Van Haastert *et al.*, 1987). When cells were forced to enter the multicellular stage by treating them with pulses of cAMP during starvation, slugs were formed with an altered proportion of prespore and prestalk region, which developed in very small fruiting bodies (Wang and Schaap, 1985).

Recently, mutant HB3 was described which has a somewhat similar phenotype as mutant *synag 7*. HB3 cells aggregate without stream formation and cell aggregates form multiple tips, which develop into small and abnormally proportioned fruiting bodies (Barclay and Henderson, 1986). Here we describe that this mutant also shows a strongly reduced cAMP relay response. In contrast to mutant *synag 7*, which does not make cAMP in response to cAMP, mutant HB3 produces normal levels of intracellular cAMP, however, it is poorly secreted. The results suggest that mutant HB3 has a defect in the cAMP secretion mechanism. Furthermore, the results suggest that extracellular cAMP rather than intracellular cAMP is an important regulator of cell differentiation.

MATERIALS AND METHODS

Materials

[2,8-^{3}H]cAMP and the cGMP radioimmunoassay kit were obtained from Amersham; dcAMP[1]) and dithio-

[1] Abbreviations used: dcAMP, 2'-deoxyadenosine 3':5'-monophosphate; (Sp)-cAMPS, adenosine 3':5'-monophosphorothioate, Sp-isomer.

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threitol were from Sigma. cAMP and (Sp)-cAMPS were purchased from Boehringer-Mannheim. Mutant HB3 was kindly provided by Drs. Barclay and Henderson.

Culture Conditions

Mutant HB3 and wild-type NC4 were grown in association with *Escherichia coli* on buffered glucose peptone agar (Van Haastert and Van der Heijden, 1983). Cells were harvested in the late-logarithmic phase with 10 m*M* KH_2PO_4/Na_2HPO_4 pH 6.5 (PB). Bacteria were removed by repeated centrifugations at 100*g* for 2 min, and cells were starved for 5 hr by shaking in PB at a density of 10^7 cells/ml or by plating on nonnutrient agar. Then, cells were collected by centrifugation, washed twice in PB, and resuspended in this buffer at a density of 10^8 cells/ml.

cAMP Synthesis and Secretion

The accumulation of intracellular and extracellular cAMP was measured with the dcAMP-method (Van Haastert, 1984). Briefly, about 3 ml of a cell suspension were stimulated with 5 μ*M* dcAMP and 10 m*M* dithiothreitol. Samples for the determination of total cAMP or extracellular cAMP were taken from the same batch of cells: simultaneously, 100-μl aliquots of the cell suspension were added to 100 μl perchloric acid (total cAMP), and 120-μl aliquots were briefly centrifuged (about 5 sec at 10,000*g*) and 100 μl of the supernatant were added to 100 μl perchloric acid (extracellular cAMP). After neutralization of the lysates, the cAMP content was determined by a sensitive isotope-dilution assay.

Surface cAMP Binding

Binding of cAMP to cells was determined in an incubation mixture of 100 μl containing PB, 10 m*M* dithiothreitol, different concentrations of [^{3}H]cAMP, and 8 $\times 10^6$ cells in a total volume of 100 μl. Equilibrium binding was determined after an incubation period of 45 sec at 20°C by centrifugation of the cells through a layer of silicon oil (Van Haastert and De Wit, 1984). The cell-associated radioactivity in the pellet was determined. Binding to the slowly dissociating sites was estimated by the addition of 10 μl 0.1 m*M* cAMP at 45 sec after the onset of the binding reaction, and centrifugation of the cells 10 sec thereafter. Nonspecific binding was determined by including 0.1 m*M* cAMP during the entire incubation period.

Other Assays

The cAMP-induced cGMP accumulation was detected by using a radioimmunoassay as described (Van Haastert and Van der Heijden, 1983). Chemotaxis was analyzed with the small population assay (Konijn, 1970).

RESULTS

Surface cAMP Receptors

Equilibrium binding of cAMP to mutant HB3 and wild-type NC4 cells is shown as a Scatchard plot in Fig. 1. We have recently proposed that *D. discoideum* cells possess subpopulations of binding sites which show different dissociation rates of bound cAMP. Scatchard plots at equilibrium, shown in Fig. 1A, are the result of cAMP binding to the whole population of binding sites, i.e., fast dissociating receptors with high- and low-affinity sites (H and L, respectively) and the slowly dissociating receptors (Van Haastert *et al.*, 1986). Scatchard plots of the slowly dissociating sites demonstrate that there are no significant differences between wild-type and mutant cells (Fig. 1B). Subtraction of these data from equilibrium binding yields the binding to H + L (Fig. 1A, dashed lines).

These results indicate that mutant cells have significantly less binding sites than wild-type cells as a consequence of reduced levels of the fast dissociating binding class.

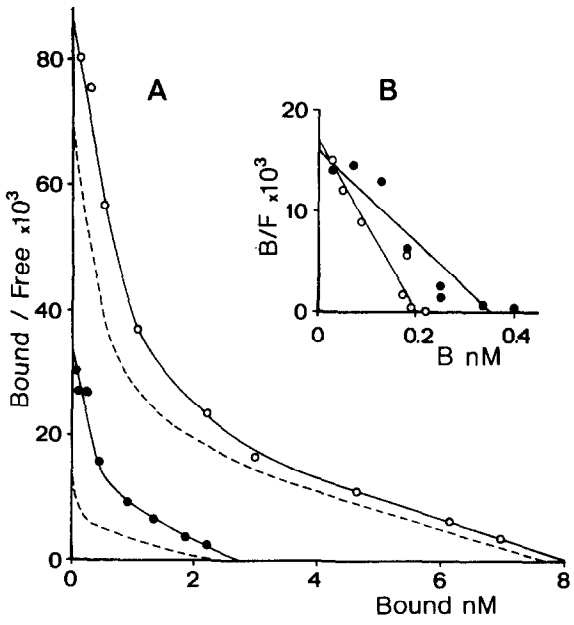

FIG. 1. Scatchard plot of cAMP-binding to wild-type cells (○) and mutant HB3 (●). The binding of 2, 5, 10, 30, 100, 200, 500, 1000, and 2000 n*M* [^{3}H]cAMP was measured at binding equilibrium (A) or at 10 sec after a chase with 0.1 m*M* cAMP (B) as described under Materials and Methods. A Scatchard plot of the slowly dissociating sites at the start of the cAMP chase was calculated and subtracted from total binding at equilibrium (dashed lines in the main figure). The results shown are the means of two (○) or three (●) independent experiments, each in triplicate.

Chemotaxis

Chemotaxis of wild-type and mutant cells to cAMP was determined with the small population assay (Konijn, 1970). Small droplets containing about 500 cells were deposited on the surface of hydrophobic agar, and small droplets containing different cAMP concentrations were deposited close to the amoeba populations just before the onset of aggregation. The distribution of cells was observed during 1 hr at 5-min intervals. A positive chemotactic response was defined when at least twice as many cells were pressed against the edge close to the cAMP than against the opposite edge (Fig. 2B). *Dictyostelium* cells do not respond with a positive chemotactic reaction at very high cAMP concentrations, but cells are pressed against the entire boundary of the population; this reaction is addressed as a centrifugal response (Fig. 2C). In wild-type cells cAMP concentrations between 10^{-9} and 10^{-6} *M* induce a positive chemotactic response and higher concentrations induce a centrifugal response (Fig. 2D). Mutant HB3 cells show a positive chemotactic response at concentrations at and below 10^{-8} *M*, and react with a centrifugal reaction above 10^{-8} *M* cAMP (Fig. 2E).

cAMP-Induced cGMP Response

cAMP induces the rapid accumulation of cGMP levels, which reach maximal levels after about 10 sec. cGMP levels return to basal concentrations within about 30 sec. The cGMP response of wild-type and mutant cells are essentially the same (Fig. 3).

cAMP-Induced cAMP Response

In preliminary experiments the accumulation of total cAMP levels was measured after stimulation of cells with dcAMP in the presence of the phosphodiesterase inhibitor dithiothreitol (Fig. 4). Total cAMP levels increase in mutant and wild-type cells to similar levels. However, the cAMP concentration declines thereafter in mutant cells, while in wild-type cells the cAMP concentration remains high. The observed differences between the responses in mutant and wild-type cells encouraged us to measure simultaneously the total and extracellular cAMP concentration. Such experiments allow the calculation of the intracellular cAMP concentration and the secretion rate (Van Haastert, 1984). The experimental results are shown in Fig. 5.

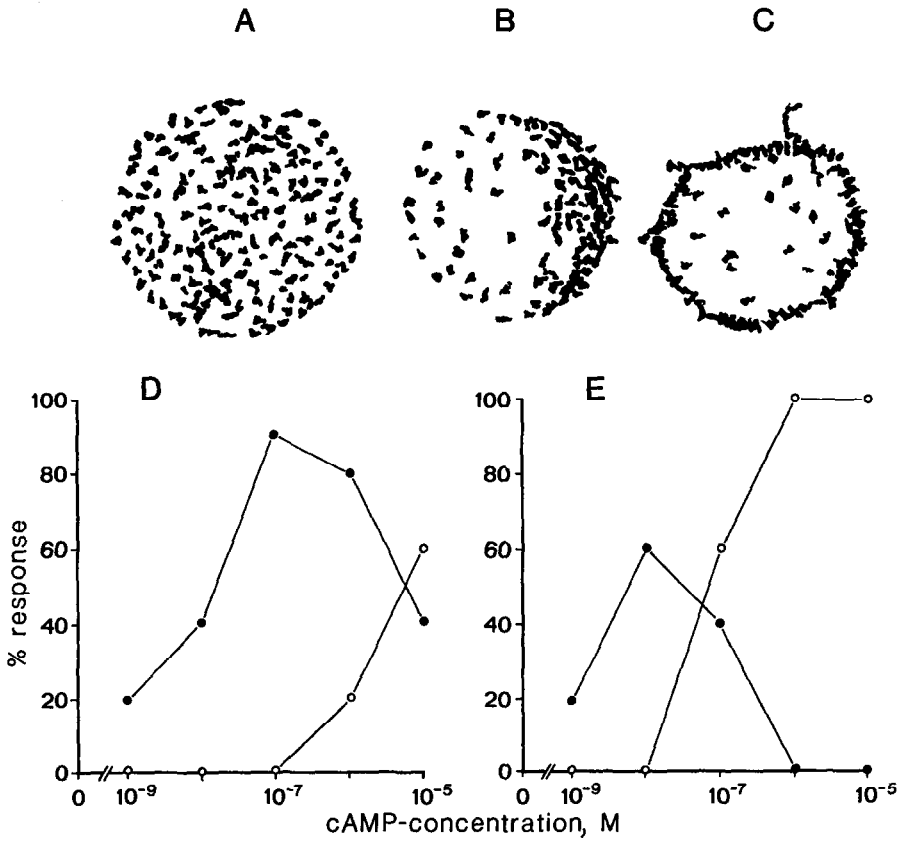

FIG. 2. Chemotaxis in wild-type and mutant cells. Distribution of wild-type cells in droplets on hydrophobic agar. (A) Control; (B) positive response to 10^{-8} *M* cAMP (placed at right site); (C) centrifugal response to 10^{-6} *M* cAMP (placed at right site). (D, E) Positive (●) and centrifugal response (○) of wild-type cells (D) and mutant HB3 (E) to different cAMP concentrations.

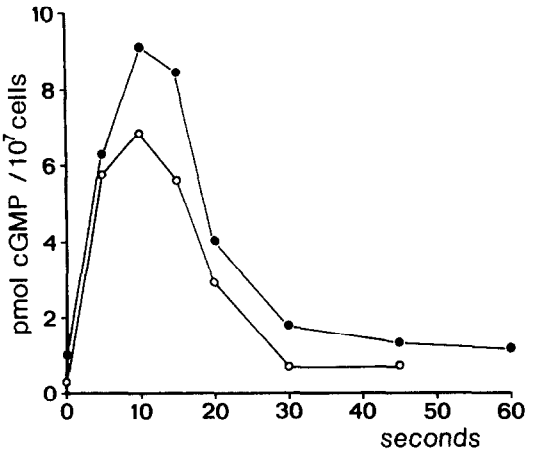

FIG. 3. Cyclic GMP accumulation after stimulation of wild-type cells (O) or mutant HB3 (●) with 100 n*M* cAMP. The results are the means of duplicate determinations of a representative experiment, which was reproduced three times.

In wild-type cells (Fig. 5A), intracellular cAMP levels increase after about 30 sec, are maximal after about 2 min, and then decline to basal levels. Secretion of cAMP starts immediately after the rise of intracellular cAMP, has a maximal rate after about 2 min, and is completed after about 4 min. During the entire period when cAMP is secreted (between 1 and 4 min), the rate of cAMP secretion is proportional to the intracellular cAMP concentration. These observations are very similar to those published previously (Dinauer *et al.*, 1980a; Van Haastert, 1984).

The cAMP response of mutant HB3 is quite different (Fig. 5B). First, intracellular cAMP levels start to increase more rapidly, reaching maximal levels after about 1 min. Second, the decline of intracellular cAMP is very rapid with basal levels recovered within about 2 min. Third, there is almost no cAMP secreted (Fig. 5B). The kinetics of the small amount of cAMP secretion show that there is essentially no secretion when the intracellular cAMP concentration is high, and secretion starts when intracellular cAMP has decreased already considerably. As a result, the amount of cAMP secreted is less than 10% of that in wild-type cells.

The early decline of intracellular cAMP in mutant HB3 can be due to several mechanisms, including a more rapid adaptation of adenylate cyclase and a more active degradation by intracellular phosphodiesterase.

Desensitization of Adenylate Cyclase

The kinetics of adaptation of adenylate cyclase in wild-type and mutant cells is shown in Fig. 6. Cells were prestimulated with the nonhydrolyzable analog (Sp)-cAMPS for different time periods, the cells were washed and challenged with the stimulus, and cAMP levels were measured after 55 sec and 5 min in mutant and wild-type cells, respectively. The results indicate that desensitization of adenylate cyclase is not faster in mutant HB3 than in wild-type cells.

Intracellular Degradation of cAMP

The rate of intracellular degradation of cAMP can be easily measured with the experiment shown in Fig. 7, which uses the notion that NaN_3 does not only block the accumulation of cAMP (Dinauer *et al.*, 1980a) but also its secretion. The experiment was performed at 0°C, because high intracellular cAMP levels accumulate in wild-type cells at this lowered temperature (Van Haastert, 1984). Figure 7A indicates that intracellular cAMP is degraded with a half-life of 1.8 min in wild-type cells. This half-life is 2.6 min in mutant HB3 (Fig. 7B). These results indicate that the rapid decline of intracellular cAMP is not due to an earlier desensitization of adenylate cyclase activation, nor to a more rapid degradation by intracellular phosphodiesterase.

DISCUSSION

D. discoideum is a eukaryotic microorganism with a relatively small haploid genome, making this organism suitable for the isolation and genetic and biochemical analyses of mutants. We have started with the analysis of transmembrane signal transduction in various mutants which possess surface cAMP receptors but show abnormal development. In this report we describe mutant HB3 which was isolated by Barclay and Henderson (1986), who showed that the mutant makes cAMP receptors, aggregates poorly with multiple tips, and develops to very small fruiting bodies with an altered proportion of stalk and spore cells. Previously, we have

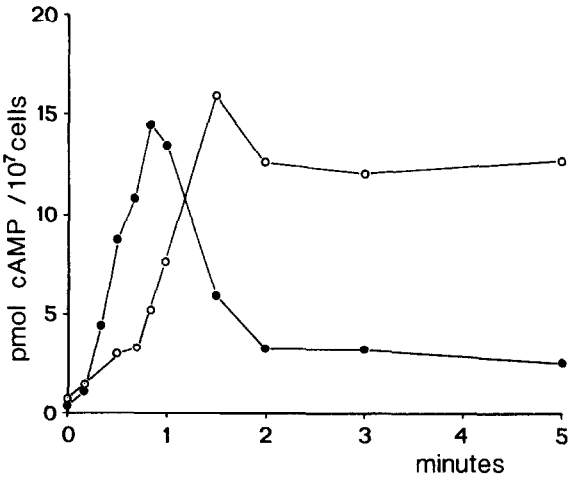

FIG. 4. Cyclic AMP accumulation after stimulation of wild-type cells (O) and mutant HB3 (●) with 5 μ*M* dcAMP and 5 m*M* dithiothreitol. No discrimination was made between extracellular and intracellular cAMP. The results are the means of duplicate determinations of a representative experiment, which was reproduced five times.

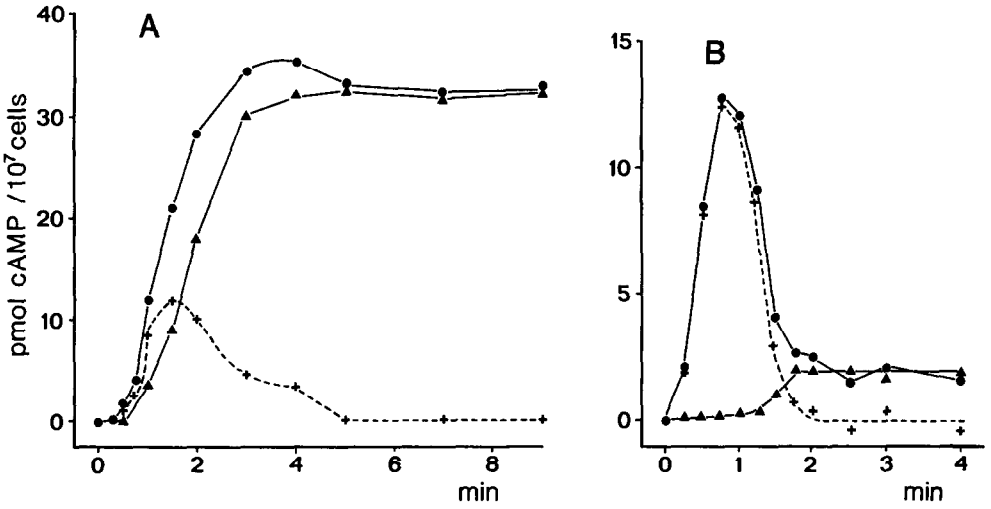

FIG. 5. Intracellular accumulation and secretion of cAMP after stimulation of wild-type (A) and mutant HB3 cells (B). Cells were stimulated with 5 μM dcAMP and 5 mM dithiothreitol and simultaneously aliquots were withdrawn for the determination of total (●) and extracellular cAMP (▲) (see Materials and Methods). From these data the intracellular cAMP concentration was calculated (+). The results are the means of three (A) or two (B) independent experiments.

shown that the surface receptors of *D. discoideum* cells are composed of two subpopulations which show different rates of dissociation of bound cAMP (Kesbeke and Van Haastert, 1985; Van Haastert *et al.*, 1986). Mutant HB3 has normal levels of the slowly dissociating cAMP receptors, but reduced levels of the fast dissociating subpopulation. We did not obtain evidence for increased levels of cAMP binding activity or for binding sites with a reduced affinity, as was described for this mutant (Barclay and Henderson, 1986). Mutant HB3 demonstrates a chemotactic response, which is qualitatively different from the response of wild-type cells. Low concentrations of cAMP induce a positive chemotactic response in wild-type and mutant cells. cAMP concentrations above 10^{-8} M lead to an undirected response in mutant HB3, which is observed in wild-type cells only above 10^{-7} M. Apparently, mutant HB3 cells detect extracellular cAMP, but cannot effectively determine or respond to the chemotactic gradient of cAMP. These observations could explain the altered aggregation pattern in this mutant.

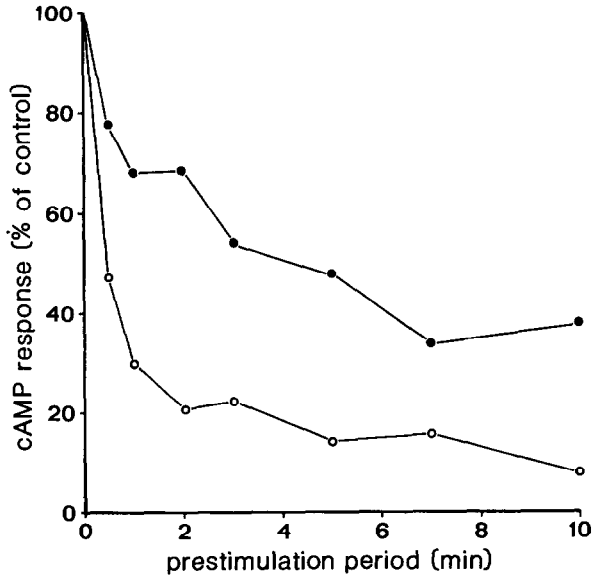

FIG. 6. Adaptation kinetics of adenylate cyclase in wild-type (○) and mutant HB3 cells (●). Cells were prestimulated with 10 μM (Sp)-cAMPS for the times indicated, extensively washed, and restimulated with dcAMP and dithiothreitol; cAMP levels were measured at 5 min and 55 sec after stimulation of wild-type and mutant cells, respectively. The results are the means of two independent experiments.

The analysis of mutant *stm* F suggests that the cAMP-induced cGMP accumulation is implicated in the chemotactic response (Ross and Newell, 1981). We did not observe a defect in the cAMP-induced cGMP accumulation in mutant HB3 cells. Signal transduction in mutant *synag 7* (previously named N7) indicates that the cAMP-induced cAMP accumulation is not essential for the induction of a chemotactic response (unpublished observations). Nevertheless we observed that the cAMP response is strongly altered in mutant HB3. This suggests that the primary defect in HB3 alters at least two components of the signal transduction pathway (chemotaxis and cAMP response) which do not show a causal relation. The defective cAMP accumulation was studied in some detail in mutant HB3.

The cAMP-induced accumulation of extracellular cAMP (relay) is a complex process. Binding of cAMP to surface receptors induces the activation of adenylate cyclase, probably via a G protein (Theibert and Devreotes, 1986; Van Haastert *et al.*, 1987). The activation of adenylate cyclase declines after a few minutes by an adaptation process, even when the stimulus persists.

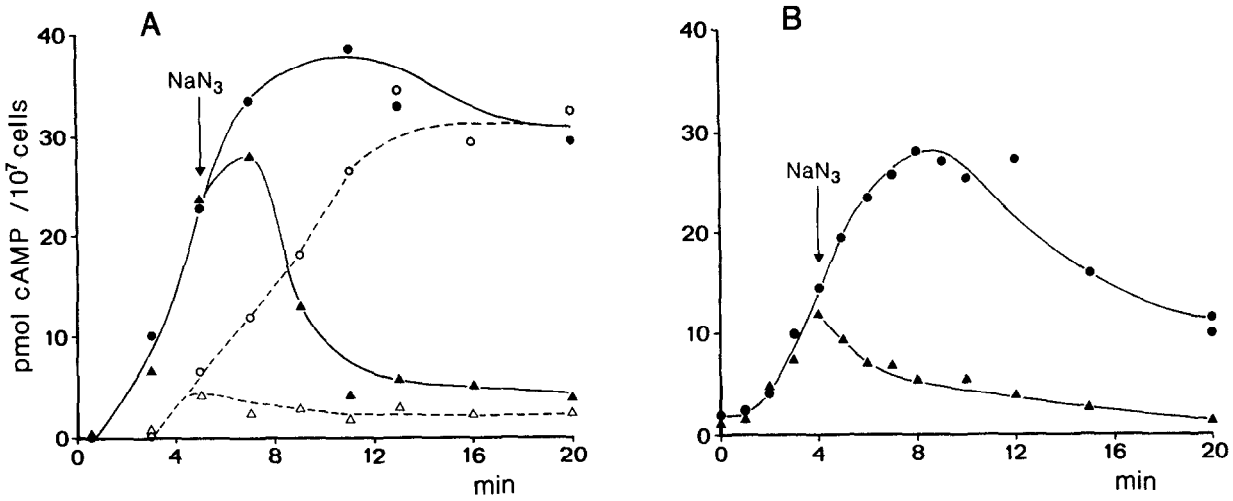

FIG. 7. Degradation rates of intracellular cAMP in wild-type (A) and mutant HB3 cells (B). Cells were stimulated at 0°C with dcAMP and dithiothreitol, and samples were taken for the determination of total (●) and extracellular (○) cAMP. Parts of the cell suspensions were challenged with 0.1 m*M* NaN_3 at 4 min after stimulation. This drug blocks the further accumulation (▲) and secretion (△) of cAMP. Thus the decrease of total cAMP represents the rate of intracellular cAMP degradation. The results are the means of two independent experiments.

The synthesized cAMP is either degraded intracellularly or secreted. Dinauer *et al.* (1980a) have shown that about 50% of the synthesized cAMP is degraded at 20°C with a rate constant of about 1.7 min^{-1} in wild-type cells. Using another method we find at 0°C a rate constant of 0.46 min^{-1}, which agrees well with the twofold lower activity of soluble phosphodiesterase activity at 0°C if compared to 20°C (unpublished results). cAMP induces the activation of adenylate cyclase in mutant HB3, leading to an increase of intracellular cAMP to a level comparable to that in wild-type cells. However only a very small portion of this cAMP is found extracellularly in mutant HB3; secretion is delayed in such a way that intracellular cAMP is almost completely degraded before secretion starts. We could not find a biochemical reason for the enhanced decrease of intracellular cAMP in mutant HB3. The rate of intracellular cAMP degradation and the apparent rate constant of desensitization of adenylate cyclase are not increased in the mutant. This probably indicates that the secretion mechanism itself is defective in mutant HB3. Since little is known about the mechanism by which *D. discoideum* cells secrete cAMP, we have not been able to trace the biochemical defect any further.

Although we have not been able to localize the biochemical defect in mutant HB3, the defect in cAMP secretion could be very helpful for investigations of the role of intracellular cAMP in differentiation, especially if this mutant is used in combination with mutants *synag 7* and HSB1 (Wang and Schaap, 1985; Bozzaro *et al.*, 1987). These mutants show some aspects of chemotaxis and have a cGMP response, but no cAMP relay. There is ample evidence that cAMP regulates development in *D. discoideum* (Gerisch, 1987). Wang *et al.* (1988) recently demonstrated that removal of extracellular cAMP inside slugs, by adding cAMP-phosphodiesterase linked to sepharose beads, induced dedifferentiation of prespore cells in *D. discoideum.*

Whereas mutant HB3 makes cAMP but does not secrete it, mutant *synag 7* and HSB1 are unable to make cAMP in response to cAMP. cAMP induced prespore- and preaggregative-gene expression is essentially normal in mutant *synag 7* and HSB, respectively.

All these data suggest that extracellular rather than intracellular cAMP directs development in *Dictyostelium discoideum.*

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